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An Unusual Mosaic Protein with a Protease Domain, Encoded by the *nudel* Gene, Is Involved in Defining Embryonic Dorsoventral Polarity in Drosophila

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Summary

Dorsoventral polarity of the Drosophila embryo is induced by a ventral extracellular signal, which is produced by a locally activated protease cascade within the extraembryonic perivitelline compartment. Local activation of the protease cascade depends on a positional cue that is laid down during oogenesis outside the oocyte. Here we present evidence that the *nudel* gene encodes an essential component of this cue. The *nudel* gene, which is expressed in follicle cells covering the oocyte, encodes an unusual mosaic protein resembling an extracellular matrix protein with a central serine protease domain. Our findings suggest that embryonic dorsoventral polarity is defined by a positional cue that requires the nudel protein to anchor and to trigger the protease cascade producing the polarity-inducing signal.

Introduction

Embryonic dorsoventral polarity in Drosophila is determined by positional information that originates outside of the embryo. This information is created by follicle cells, the somatic tissue surrounding the oocyte responsible for secreting eggshell components (Schüpbach, 1987; Stein et al., 1991). The follicle cells may create a spatial asymmetry in the eggshell that is utilized after fertilization to polarize the embryo. This unusual mechanism for defining embryonic polarity has been referred to as delayed induction, as the positional information created by the follicle cells is transmitted long after these cells have degenerated toward the end of oogenesis (St Johnston and Nüsslein-Volhard, 1992).

The external spatial asymmetry is conveyed to the embryo through a signal transduction pathway involving maternal gene products (Morisato and Anderson, 1995). A ventrally restricted signal is first generated within the perivitelline space between the embryo and the vitelline envelope, the innermost layer of the eggshell. This signal acts as a ligand for the membrane receptor Toll, which is uniformly distributed on the embryonic surface (Hashimoto et al., 1988, 1991; Stein et al., 1991). Ventral activation of Toll ultimately leads to differential localization of the dorsal protein in nuclei of the syncytial embryo (Roth et al., 1989; Rushlow et al., 1989; Steward, 1989). The graded nuclear distribution of dorsal, a transcriptional regulator of the *rel*/NF-κB family, specifies the regional expression of zygotic gene products that function to elaborate the dorsoventral pattern of the embryo (Govind and Steward, 1991).

An important question is how the signal that functions as the ligand of Toll is ventrally localized. Among the maternal genes required to produce the Toll ligand, *spätzle* is most immediately upstream of *Toll* and therefore likely encodes the ligand (Morisato and Anderson, 1994). Proteolytic processing of the spätzle protein to produce the active form of the Toll ligand requires the products of the genes *gastrulation defective*, *snake*, and *easter* (Morisato and Anderson, 1994; Schneider et al., 1994). These gene products are structurally similar to zymogens of serine proteases that require proteolytic cleavage to become active (DeLotto and Spierer, 1986; Chasan and Anderson, 1989; K. Konrad and L. Marsh, personal communication). Like the enzymes of mammalian blood clotting, they may be sequentially activated in a cascade (Chasan et al., 1992; Smith and DeLotto, 1994). Transplantation and biochemical experiments suggest that the snake and easter zymogens and the spätzle protein are freely diffusible within the perivitelline space (Stein and Nüsslein-Volhard, 1992; Chasan et al., 1992; Morisato and Anderson, 1994). Thus, activation of the proteases necessary for spätzle processing must be localized to limit production of the Toll ligand to the ventral side of the embryo (Chasan et al., 1992; Smith and DeLotto, 1994).

Three genes, *nudel*, *pipe*, and *windbeutel*, are required to generate positional information that directs the ventral formation of the Toll ligand. Embryos derived from mothers mutant for any of these genes completely lack dorsoventral polarity, apparently because the protease cascade leading to the Toll ligand fails to be activated (Stein et al., 1991; Stein and Nüsslein-Volhard, 1992; Chasan et al., 1992; Morisato and Anderson, 1994; Schneider et al., 1994; Smith and DeLotto, 1994). Experiments with genetically mosaic females indicate that embryonic dorsoventral polarity requires the activities of these genes in somatic tissue, presumably the follicle cells that secrete eggshell components around the oocyte during oogenesis (Schüpbach et al., 1991; Stein et al., 1991). These observations suggest that the *nudel*, *pipe*, and *windbeutel* gene products function to generate a ventrally localized component of the vitelline envelope that directs the local activation of the protease cascade producing the Toll ligand.

Here we present genetic and molecular evidence that the *nudel* gene product is directly involved in locally producing the Toll ligand. We cloned the *nudel* gene and found that it is expressed in follicle cells when they are secreting vitelline envelope components during oogenesis. The *nudel* gene encodes a large modular protein that structurally resembles extracellular matrix proteins. Strikingly, within the center of its primary structure is a serine protease domain likely to be catalytically active. We propose that the nudel protein is required both to anchor and to trigger the protease cascade producing the Toll ligand and is therefore an essential component of the ex-

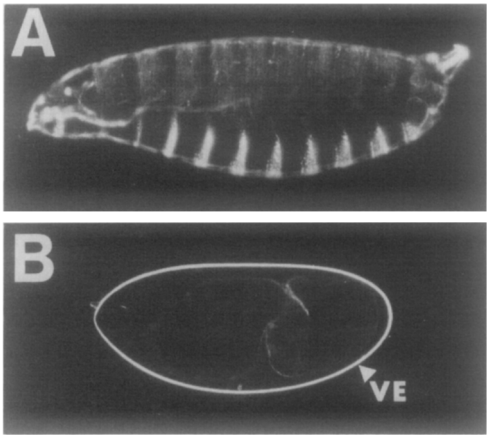

Figure 1. Dorsalized Cuticle Phenotype Produced by *nudel* Mutant

(A) Cuticle of larva from wild-type female (anterior, left; dorsal, top). Dorsoventral pattern elements include ventral denticle belts, lateral structures of head and tail, and dorsal hairs.

(B) Dorsalized cuticle produced by embryo from ndl^{46}/ndl^{46} female. The cuticle is twisted and lacks ventral and lateral structures. The dorsalized embryo, which fails to hatch, is contained within the vitelline envelope (VE).

tracellular cue that defines embryonic dorsoventral polarity.

Results

Dorsalized Embryos and Fragile Eggs Produced by *nudel* Mutants

Females lacking normal *nudel* activity produce embryos that become dorsalized. The cuticle formed by dorsalized embryos mostly contains structures normally restricted to the dorsal surface of the wild-type cuticle (Figure 1A and B). In addition to becoming dorsalized, eggs produced by *nudel* mutants are unusually fragile and therefore very sensitive to manipulation (Anderson and Nüsslein-Volhard, 1986). This additional phenotype has not been described for mutations in the two other somatically required genes, *pipe* and *windbeutel* (Anderson and Nüsslein-Volhard, 1986; Schüpbach et al., 1991). Most but not all *nudel* mutant alleles produce the fragile egg phenotype, which is particularly apparent when eggs are only covered by the vitelline envelope and not the entire eggshell (our unpublished data). Thus, the *nudel* gene product could be a component of the vitelline envelope required for structural integrity of the egg.

Temperature-Sensitive Period for *nudel* Function

Since embryonic dorsoventral polarity depends on maternal *nudel* activity in somatic tissue, the *nudel* gene product could function during oogenesis. Yet if the *nudel* gene product is a component of the vitelline envelope, *nudel* function could also be required after fertilization. To examine when *nudel* function might be required for establishing polarity, we carried out temperature shift experiments using heat-sensitive *nudel* alleles to regulate *nudel* activity during ovarian and embryonic development (see Experimental Procedures). As shown in Figure 2A, the temperature-sensitive period for *nudel* function begins roughly at stage 7 of oogenesis, just before the beginning of yolk deposition and the expression of genes encoding vitelline envelope proteins by the follicle cells (Spradling, 1993). Most importantly, as shown in Figure 2B, the temperature-sensitive period for *nudel* function ends roughly 2 hr after fertilization. Thus, *nudel* function is required not just during oogenesis but also during early stages of embryogenesis.

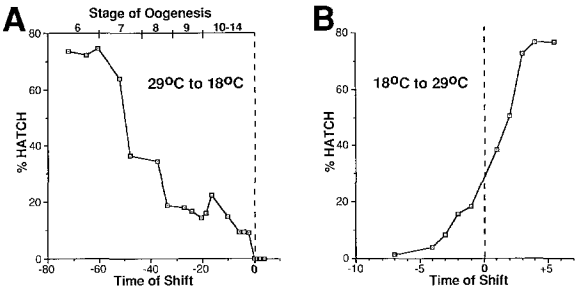

Figure 2. Temperature-Sensitive Period for *nudel* Function

Graphs show the percentage of eggs laid by ndl^{9}/ndl^{46} females that hatch following temperature shifts at indicated times.

(A) Shift from nonpermissive to permissive temperature. The temperature-sensitive period for *nudel* begins around stage 7 of oogenesis, just before follicle cells begin expressing genes encoding vitelline envelope proteins. Top abscissa shows oogenic stages corresponding to times of shift.

(B) Shift from permissive to nonpermissive temperature. The temperature-sensitive period for *nudel* ends roughly 2 hr after fertilization, suggesting that *nudel* is also required during early embryogenesis.

Cloning of the *nudel* Gene and Its Expression in Ovarian Follicle Cells

The fragile egg phenotype and the temperature shift experiments suggested that the *nudel* gene product could be present during early embryogenesis to act directly in producing the Toll ligand. To understand how the *nudel* gene product functions biochemically in this process, we decided to clone the *nudel* gene. Previously, *nudel* had been localized by meiotic recombination to position 3-17 in the left arm of the third chromosome (Anderson and Nüsslein-Volhard, 1984). To localize *nudel* in the polytene chromosome map, we generated deficiencies within the *nudel* region by X-ray mutagenesis (see Experimental Procedures). By complementation analyses using these deficiencies and a deficiency associated with a P transposable element insertion, we localized *nudel* to 65B5–65C1 (Figure 3A). We isolated 160 kb of DNA covering this region in a chromosome walk (see Experimental Procedures). By Southern blot analyses, we mapped the breakpoints of the deficiencies *Df(3L)CH39* and *l(3)3844* within the cloned region, which refined the location of *nudel* to within 70 kb of DNA (Figure 3B).

Since *nudel* was determined to be required in somatic tissue, it was expected to be expressed in follicle cells that cover the oocyte during oogenesis (Stein et al., 1991). We therefore tested whether the 70 kb region encodes a follicle cell–specific transcript by RNA in situ hybridization analyses of ovaries. DNA from only one segment of this

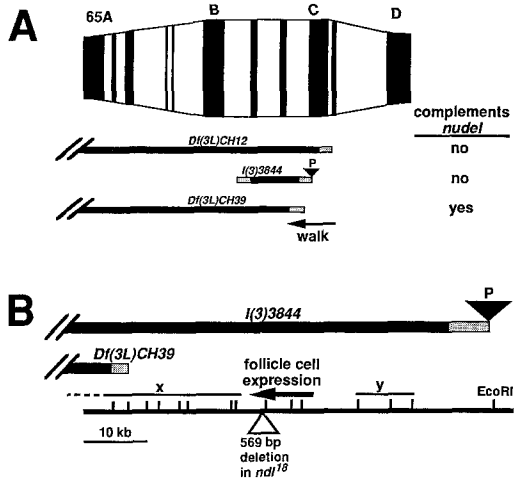

Figure 3. Cytogenetic and Molecular Maps of *nudel* Region

(A) Cytogenetic map of *nudel* locus. Top, the 65A–D region of the polytene chromosome, and below, deficiencies that localize the *nudel* locus to the 65B5–65C1 interval. Solid bars represent deleted regions, while gray bars represent uncertainties in breakpoints. A chromosome walk of 160 kb that covers the *nudel* region is represented by the arrow. (B) Molecular map of *nudel* region. Proximal breakpoints of deficiencies *l(3)3844* and *Df(3L)CH39* localize *nudel* to an interval of 70 kb within the chromosome walk. Shown below is an EcoRI restriction map of this DNA that detects three ovarian transcripts. Two (x and y) are expressed mostly in nurse cells, whereas the third (thick arrow) is expressed predominantly in follicle cells (see Figures 4A–4B). The triangle represents a 569 bp deletion in DNA of the *ndl*[18] mutant that causes truncation of the follicle cell–specific transcript (see Figure 4D).

region hybridized to RNA expressed almost exclusively in follicle cells. Expression of this RNA was first detectable at a low level in stage 7 of oogenesis but reached a peak level during stages 9 and 10, when follicle cells are known to express genes for vitelline envelope proteins (Figures 4A and 4B). Interestingly, in the majority of egg chambers, this RNA was asymmetrically distributed, with a higher level seen in ventral than in dorsal follicle cells. Although this asymmetry was moderate in 53% of stage 9 or 10 egg chambers (Figure 4A; Table 1), it was striking in about 18% of the stage 10 egg chambers (Figure 4B; Table 1). In the remaining 29%, the distribution of the follicle cell–specific RNA was not noticeably asymmetric (Table 1). This RNA decreased dramatically in level during late stage 10, becoming undetectable by stage 11. As a control and for comparison, we analyzed the distribution of the RNA encoding the vitelline envelope protein Sv23 (Popodi et al., 1988). Although this RNA was also expressed at a high level during stages 9 and 10, it was uniformly distributed in most of the egg chambers (Figure 4C; Table 1).

To determine whether the follicle cell–specific RNA that we identified represents the *nudel* transcript, we performed Northern blot analyses using poly(A)$^+$ RNA from ovaries of normal and *nudel* mutant mothers. When blots containing these RNAs were probed with DNA that detects the follicle cell–specific RNA by in situ hybridization, a single RNA of approximately 8.4 kb was detected in normal ovaries (Figure 4D). This RNA was undetectable or greatly reduced in amount in several of the strongest *nudel* mutants, and appeared to be truncated in the *nudel*[18] (*ndl*[18]) mutant. By Southern blot, polymerase chain reaction (PCR), and DNA sequence analyses, we found a deletion of 569 bp in genomic DNA from the *ndl*[18] mutant that is likely responsible for truncation of the RNA (data not shown; see Figure 3B). This deletion toward the 3′ end of the RNA is expected to cause a frameshift during translation that would truncate the single long open reading frame encoded by the 8.4 kb RNA (see below). The alterations in abundance and structure of the 8.4 kb RNA in *nudel*

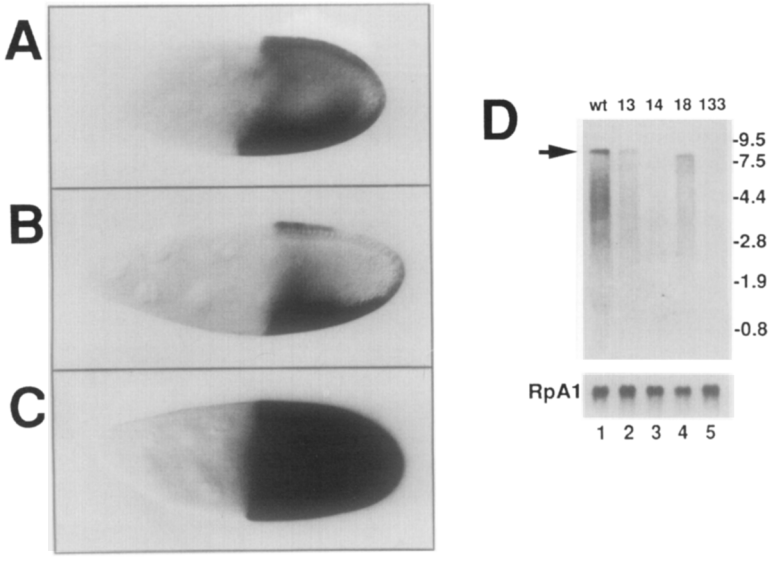

Figure 4. Identification of the *nudel* Transcript

(A–C) RNA in situ hybridization analyses of ovaries. In wild-type egg chambers, a follicle cell–specific RNA is detected by DNA from the 70 kb interval containing the *nudel* locus (see Figure 3B). More of this RNA is detectable in ventral than in dorsal follicle cells (A). This asymmetry is more pronounced in about 18% of the stained egg chambers (B). In contrast, RNA encoding vitelline envelope protein Sv23 is more evenly distributed (C). All egg chambers are at oogenesis stage 10 (anterior, left; dorsal, top). Visual localization of the nucleus in the dorsoanterior corner of the oocyte was used to determine the orientation of egg chambers (the nucleus is not visible in the optical sections shown here).

(D) Northern blot analyses of ovarian RNA. A blot containing ovarian poly(A)$^+$ RNA from normal (lane 1) and *ndl* mutant (lanes 2–5) females was probed with DNA that hybridizes to the follicle cell–specific RNA shown in (A)–(B) (5 μg RNA/lane; wt and numbers at the top represent wild-type and mutant *ndl* alleles over a *ndl* deficiency). This DNA detects a transcript of 8.4 kb (arrow) in normal ovaries that becomes truncated in the *ndl*[18] mutant (lane 4). The truncation is due to deletion of genomic DNA from the 3′ end of the transcription unit (see Figure 3B). The same blot was probed for RNA encoding ribosomal protein A1 (RpA1; Kay and Jacobs-Lorena, 1985) to provide a standard for RNA levels. Numbers at the right refer to RNA size standards in kilobases.

Table 1. Asymmetric Expression of *nudel* RNA in Ovarian Egg Chambers

Probe	Egg Chamber Genotype	Percent No Asymmetry	Percent Mild Asymmetry	Percent Strong Asymmetry	Number of Egg Chambers
nudel	Wild type	29	53	18	210
	torpedo	28	46	26	39
	fs(1)K10	66	27	7	74
	pipe	70	28	2	57
Sv23	Wild type	80	17	3	84

Expression of the *nudel* transcript or the RNA for vitelline envelope protein Sv23 in ovaries from wild-type or mutant females was detected by in situ hybridization. Differences in hybridization signal in follicle cells along the dorsoventral dimension of stage 9 or 10 egg chambers were estimated by visual examination. No asymmetry represents uniform expression in follicle cells, whereas mild or strong asymmetry, respectively, represents less than or greater than 5-fold enrichment of signal in ventral over dorsal follicle cells. The mutant genotypes were as follows: *top*^{1}/*top*^{1}, *fs(1)K10*^{1}/*fs(1)K10*^{1}, *fs(1)K10*^{13}/*fs(1)K10*^{13}, and *pip*^{664}/*pip*^{664}. The results from the two different *fs(1)K10* mutants were very similar and were therefore combined.

mutants, and its expression in follicle cells, provide strong evidence that this RNA is the *nudel* transcript.

To explore whether enrichment of the *nudel* transcript in ventral follicle cells is important for embryonic dorsoventral polarity, we analyzed *nudel* expression in egg chambers from females mutant for genes that act genetically upstream of *nudel*. These maternal genes of the *gurken–torpedo* signaling pathway, including *fs(1)K10*, are required for signaling between oocyte and follicle cells that determine embryonic dorsoventral polarity; they may act indirectly to localize activation of Toll in the embryo by spatially restricting the activity of *nudel*, *pipe*, or *windbeutel* (Schüpbach et al., 1991). Because egg chambers produced by strongly ventralizing *gurken* mutations were difficult to examine (see Experimental Procedures), we analyzed egg chambers produced by a weakly ventralizing *torpedo* mutation. In this case, the number of egg chambers with ventral enrichment and uniform expression of the *nudel* transcript was not significantly different than in the wild-type case (Table 1). In contrast, *nudel* RNA was uniformly distributed in most of the egg chambers produced by strongly dorsalizing *fs(1)K10* mutations (Table 1). A similar result was obtained with egg chambers from females homozygous for a *pipe* mutation that produces strongly dorsalized embryos (Table 1). Owing to a small sample size, we were unable to quantitate the effect caused by a *windbeutel* mutation on *nudel* expression (see Experimental Procedures). The altered distribution of *nudel* RNA in the *fs(1)K10* and *pipe* mutants suggests that the asymmetry in *nudel* expression that we detected is important for embryonic dorsoventral polarity.

The *nudel* Gene Encodes a Large Extracellular Protein

To isolate cDNAs to the *nudel* mRNA, we used genomic DNA that hybridizes to the 8.4 kb RNA as a probe to screen ovarian cDNA libraries. We isolated numerous partial cDNAs that overlap to span most of the *nudel* transcript length. A cDNA corresponding to the 5′ end of the transcript was isolated by PCR. Together, these cDNAs can encode an RNA of 8230 nt, excluding the poly(A) tail. Since this length is very close to the size of the *nudel* transcript estimated by Northern blot analysis, very little of the *nudel* RNA sequence is missing from the cDNAs that we isolated. Sequence analysis of the cDNAs revealed a single long open reading frame for a protein of 2616 amino acids or 292 kDa in molecular mass (Figure 5).

The amino acid sequence indicates that the nudel protein has the potential to be secreted. A stretch of hydropho-

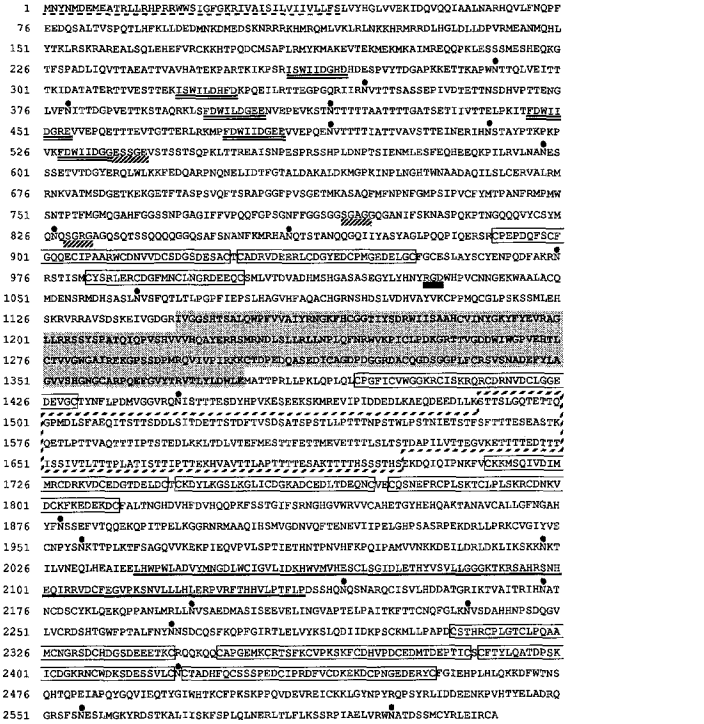

Figure 5. Amino Acid Sequence of the Nudel Protein Deduced from the cDNA Sequence

The cDNA sequence predicts a polypeptide of 2616 amino acids. The amino acid sequence begins with the first methionine after an in-frame stop codon. The putative signal sequence at the N-terminus is marked with a broken underline. Potential N-linked glycosylation sites are indicated by dots above the sequence, and potential glycosaminoglycan attachment sites are marked by hatched bars below the sequence. RGD sequence is underlined with a heavy bar. WIID repeats are double underlined. The C-terminal serine/threonine-rich domain is enclosed in a stippled box. Each LDL receptor type A repeat is boxed. Residues of the central serine protease catalytic domain are boldfaced and shaded, whereas the C-terminal protease-like domain is underlined.

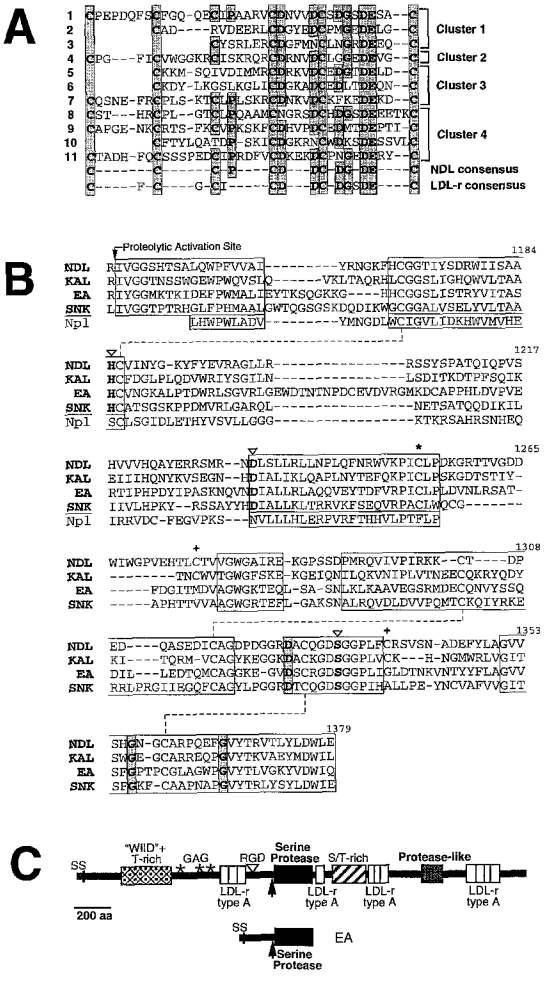

Figure 6. Nudel Is a Large Modular Protein with an Internal Serine Protease Domain

(A) Alignment of LDL receptor type A repeats of nudel. Residues found in six or more of the repeats are shown in boldface type and enclosed by stippled boxes. Gaps (dashes) were introduced to maximize alignment of cysteines. The eleven LDL receptor type A repeats are found in four clusters in the primary structure (see Figure 5). At the bottom are consensuses from nudel (NDL) and LDL receptor (LDL-r; Yamamoto et al., 1984).

(B) Alignment of protease domain (NDL) and protease-like domain (Npl) of nudel with catalytic domains of human plasma kallikrein (KAL; Chung et al., 1986), and with easter (EA; Chasan and Anderson, 1989) and snake (SNK; DeLotto and Spierer, 1986; Smith et al., 1994) proteins of Drosophila. Numbers refer to the amino acid sequence of nudel. The proteolytic activation site is indicated by arrow. Catalytic triad residues are shown in boldface type and marked by triangle. Residues that could occupy the substrate-binding pocket, as in trypsin, are enclosed in stippled boxes and shown in boldface type. Cysteines that likely form disulfide bonds, as in serine proteases of the trypsin family, are connected by broken lines. Two cysteines that probably form an additional disulfide bond are marked with crosses. The cysteine marked with a star may form a disulfide bond with a cysteine in the region N-terminal to the protease domain. Conserved regions in catalytic domains as defined by Furie et al. (1982) are enclosed in large boxes, whereas similarities between the protease-like domain of nudel (Npl) and serine protease catalytic domains are contained within smaller boxes immediately below. Gaps (dashes) were introduced to maximize alignments.

(C) Schematic of the modular structure of nudel. The serine protease catalytic domain is in the center of the primary structure of nudel, preceded by the proteolytic activation site (arrow). Nudel also has a putative N-terminal signal sequence (SS; vertical bar marks possible cleavage site), six WIID repeats in a threonine-rich region (WIID plus T-rich), three potential glycosaminoglycan attachment sites (GAG; stars), eleven LDL receptor type A repeats (LDL-r type A), the RGD sequence (triangle), a serine/threonine-rich region (S/T-rich), and a protease-like domain. In contrast with the case in nudel, the serine protease catalytic domain represents the C-terminal half of easter (EA).

bic residues near the N-terminus of the protein could be part of a signal sequence as seen in secreted and membrane-anchored proteins, since it is shortly followed by a possible signal sequence cleavage site (Figure 5; von Heijne, 1983). No other significant hydrophobic region could be found in the rest of the molecule by hydropathy analysis, so nudel is not expected to be a transmembrane protein (data not shown; Kyte and Doolittle, 1982). The nudel protein is predicted to be extensively glycosylated. There are 23 potential sites for N-linked glycosylation throughout the molecule (Figure 5) and two regions rich in serine and threonine that may be sites for O-linked glycosylation (Figures 5 and 6C; Carraway and Hull, 1991). Nudel also has three potential sites for glycosaminoglycan (GAG) attachment, as seen in proteoglycans of the extracellular matrix (Figures 5 and 6C; Krueger et al., 1990).

Nudel Is a Modular Protein with an Internal Serine Protease Domain

Like extracellular matrix proteins, the nudel protein is structurally modular, consisting of discrete motifs and domains. Two types of sequence motifs are repeated in the protein. The first, consisting of nine residues and the sequence WI(I/L)D, is a novel motif that we call the WIID repeat. Nudel contains six copies of this repeat within the N-terminal threonine-rich region (Figures 5 and 6C). The second repeated motif is a cysteine-rich sequence, consisting of about 40 residues, which was first identified in the ligand-binding domain of the low density lipoprotein (LDL) receptor (Figures 5 and 6A; Yamamoto et al., 1984). This motif, called a type A repeat, is found in a variety of proteins and is thought to mediate specific protein–protein interactions (see Discussion). The nudel protein contains 11 copies of the type A repeat in four clusters.

The most striking features of the primary structure of the nudel protein are two regions similar to the catalytic domains of extracellular serine proteases in the trypsin family (Figures 5, 6B, and 6C). The more C-terminal region is only similar to the N-terminal half of the catalytic domain. This protease-like domain lacks the residues of the catalytic triad (histidine, aspartic acid, and serine) required for enzymatic activity, so it is not likely to be active as a protease (Hartley, 1970).

The region near the middle of nudel, in contrast, is highly similar to the entire catalytic domain of a serine protease (Figures 5, 6B, and 6C). In addition to regions highly conserved in serine proteases, the catalytic triad residues are also found. Also present are eight cysteines that may form four intramolecular disulfide bonds within the catalytic pocket, as in chymotrypsin. Overall, the central protease domain of nudel is most similar to the catalytic domains

of vertebrate plasma kallikreins (34% identity; Chung et al., 1986). Three key residues in the substrate-binding pocket of trypsin that determine amino acid specificity are conserved in the protease domain of nudel, suggesting that the nudel protease will cleave after lysine and arginine (Figure 6B; Hartley, 1970).

The protease domain of nudel would presumably require proteolytic cleavage in order to become activated. Many serine proteases, such as those involved in blood clotting, are activated by cleavage at a precise site between an N-terminal regulatory region and the C-terminal catalytic domain (Furie and Furie, 1988). In nudel, an arginine is just N-terminal to the conserved IVGG sequence at the beginning of the protease domain, implying that a protease specific for basic residues activates the protease activity of nudel (Figure 6B). Upon cleavage, the catalytic domain of nudel is predicted to remain attached to the N-terminal region through a disulfide bond, as in the regulated proteases of blood clotting.

Discussion

We have characterized and cloned *nudel*, a gene maternally required in somatic tissue for ventral formation of the Toll ligand, which induces dorsoventral polarity of the Drosophila embryo. Our genetic and molecular analyses suggest that the nudel protein is a component of the positional information that is laid down during oogenesis to be used after fertilization for defining embryonic dorsoventral polarity. Because of its mosaic structure, containing diverse protein-binding motifs and a serine protease catalytic domain, nudel could function both to anchor and to trigger the protease cascade that produces the Toll ligand. We therefore propose that the nudel protein plays an essential role in defining embryonic dorsoventral polarity by acting as the spatial organizer of this protease cascade.

Nudel as a Stable Component of the Perivitelline Space

Several observations suggest that the nudel protein is a component of the perivitelline space surrounding the embryo. The *nudel* gene is most highly expressed in follicle cells of the ovarian egg chamber around the time these cells are secreting major structural proteins of the vitelline envelope (Figures 4A and 4B; Fargnoli and Waring, 1982). Since nudel is predicted to be a secreted protein (Figure 5), it could be exported like vitelline envelope components to the extracellular compartment between the follicle cells and the oocyte. Although nudel is synthesized during oogenesis, the requirement for its function during early embryogenesis suggests that it persists in the embryo (Figure 2B).

Like proteoglycans and other proteins of the extracellular matrix, we think that nudel is stabilized within the perivitelline space. That it is not freely diffusible is consistent with the inability to transplant *nudel* activity in experiments involving the transfer of perivitelline space fluid between embryos (Stein et al., 1991; Stein and Nüsslein-Volhard, 1992). How nudel might be immobilized is not yet clear. Although the fragile egg phenotype associated with most *nudel* mutations could mean that the *nudel* gene product is a structural component of the vitelline envelope, nudel is not similar to major structural proteins of the vitelline envelope. In addition to being much larger than these proteins, whose sizes are in the range of 14–24 kDa, nudel lacks a motif of 38 amino acids found in several vitelline envelope proteins (Popodi et al., 1988; Scherer et al., 1988). It is possible that nudel becomes cross-linked to structural proteins of the vitelline envelope during oogenesis or egg laying (Fargnoli and Waring, 1982). Alternatively, nudel may be noncovalently associated with the vitelline envelope via carbohydrate in O-linked or GAG chains, or the RGD sequence (D'Souza et al., 1991; Gould et al., 1992).

Nudel as a Protease

The serine protease domain in the center of its primary structure suggests that nudel functions as a protease. This conclusion is supported by our preliminary DNA sequence analyses of *nudel* mutations. In the protease domain region of the ndl^{111} allele, the highly conserved glycine just two residues C-terminal to the active site serine is changed to an arginine. Although we do not know if there are other changes in the ndl^{111} allele, this change in the protease domain is likely to impair catalytic activity. Interestingly, ndl^{111} causes embryos to become completely dorsalized but does not produce an obvious fragile egg phenotype (our unpublished data). This uncoupling of the two phenotypes associated with *nudel* mutations suggests that the nudel protein has two distinct functions: one, to provide structural integrity to the egg; and the other, to act as a protease directly involved in defining embryonic dorsoventral polarity.

The size and structure of nudel are highly unusual for a serine protease zymogen. Whereas easter and typical zymogens of blood clotting are in the range of 40–80 kDa in mass, nudel is expected to be greater than 300 kDa in mass with glycosylation (see Figure 6C for comparison). Moreover, in all known regulated serine proteases of the trypsin superfamily, the catalytic domain represents the C-terminal half of the zymogen (Figure 6C; Furie and Furie, 1988). A single proteolytic cleavage between an N-terminal regulatory region and the catalytic domain is sufficient to release protease activity. In contrast, the serine protease catalytic domain is found in the center of the primary structure of nudel, raising the possibility that two proteolytic cleavages flanking this domain are required to activate the protease function of nudel.

An interesting question is what molecule activates the protease function of nudel. Just before the IVGG sequence at the N-terminus of the protease domain of nudel is an arginine residue, indicating that a protease specific for basic residues is responsible for cleavage at this position (Figure 6B). It is possible that a gene not yet identified encodes such a protease. We think an intriguing possibility, given that the protease domain of nudel is predicted to be specific for basic residues, is that nudel is autoactivating. There are several examples of serine proteases

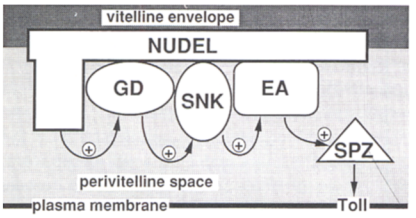

Figure 7. Model Depicting the Role of Nudel as Anchor and Trigger of the Protease Cascade That Produces the Toll Ligand

Nudel is fixed within the perivitelline space by association with the vitelline envelope. It acts as the scaffold of a zymogen activation complex containing gastrulation defective (GD), snake (SNK), and easter (EA). Its own serine protease domain, perhaps autoactivated with the help of cofactors not depicted here, cleaves gastrulation defective, thus initiating the protease cascade that ends with the proteolytic processing of spätzle (SPZ) to produce the Toll ligand (arrow marked with a plus indicates proteolytic activation). The sequential action of gastrulation defective, snake, easter, and spätzle shown here is supported by genetic studies but has not yet been biochemically demonstrated (Chasan et al., 1992; Smith and DeLotto, 1994).

whose activities arise through autoactivation by the zymogens, which can have weak proteolytic activities (Gertler et al., 1974; Nebes and Jones, 1991). The proteases at the beginning of blood clotting cascades can also be autoactivated upon incorporation into surface-bound complexes that include cofactors and substrates (Wiggins and Cochrane, 1979).

Nudel as a Spatial Determinant of Embryonic Dorsoventral Polarity

For a spatial determinant to localize the protease cascade that produces the Toll ligand, it should be capable of confining the protease activities of snake and easter, which appear to circulate as proenzymes in the perivitelline space (Stein and Nüsslein-Volhard, 1992; Chasan et al., 1992). The nudel protein could fulfill this role by organizing the assembly of a macromolecular complex that includes snake and easter as well as gastrulation defective (as proposed by Chasan et al., 1992). Upon assembly into such a complex, the proteins gastrulation defective, snake, and easter would become sequentially activated, the cascade being triggered by the protease activity of nudel itself (Figure 7). In mammals, the serine proteases of blood clotting that circulate in serum as zymogens become activated in membrane-bound macromolecular complexes (Furie and Furie, 1988). Formation of a zymogen activation complex appears to be necessary for generating maximal protease activities as well as a mechanism for localizing proteases.

The large size of the nudel protein and its modular structure appear ideal for a role as the scaffold of a zymogen activation complex (Figures 6C and 7). In addition to its central protease domain, nudel contains two other structural motifs that could be used to bind proteases or their zymogens and to facilitate the formation of a macromolecular complex. One is the LDL receptor type A motif found repeated in four separate regions (Figure 6C). Repeats of this cysteine-rich motif are present in the LDL receptor–related protein, a huge and multifunctional receptor involved in endocytosis of several different ligands, including tissue plasminogen activator (a serine protease) as well as complexes of proteases and their inhibitors (Herz et al., 1988). The LDL receptor type A repeat is also present in several components of the complement cascade that assemble to form the terminal cytolytic complex on cell membranes (Esser, 1994). The other motif is the protease-like domain near the C-terminus (Figure 6C). Although this domain is not likely to have proteolytic activity, it may still be capable of binding a substrate like an active protease.

If nudel acts as both anchor and activator of proteases, ventral localization of its protease activity could be responsible for local production of the Toll ligand. Localization of the protease activity of nudel may depend on asymmetric distribution of the protein or asymmetric activation of protease activity. In most wild-type egg chambers, *nudel* RNA is expressed at a higher level in ventral than in dorsal follicle cells (Figures 4A and 4B; Table 1). Thus, asymmetric distribution of the nudel protein may be important for defining embryonic dorsoventral polarity. Support for this idea is that strongly dorsalizing mutations in *pipe* and in *fs(1)K10*, which is required to localize *gurken–torpedo* signaling (Neuman-Silberberg and Schüpbach, 1993), significantly increase the number of egg chambers with uniform *nudel* expression (Table 1). Only mutations that strongly disrupt embryonic dorsoventral polarity seem to affect *nudel* expression. This observation may explain why the strongest available *torpedo* mutation that specifically disrupts embryonic dorsoventral polarity does not obviously alter *nudel* expression (Table 1), as this allele only produces weakly ventralized embryos that retain dorsoventral asymmetry (Schüpbach, 1987). Our RNA in situ hybridization analyses suggest that the *pipe* gene product functions to elevate *nudel* expression in follicle cells above a uniform basal level and that the *gurken–torpedo* signaling pathway acts to repress this elevation specifically in dorsal follicle cells, perhaps by inhibiting *pipe* activity.

In light of these results, we consider the following model to explain how the production of the Toll ligand is ventrally localized. A modest gradient of the nudel protein, with the highest level ventrally, is established within the perivitelline space as a result of the *gurken–torpedo* signaling pathway during oogenesis. The amount of nudel directly determines the level of zymogen activation complex that is formed. The protease activity of nudel, either autoactivated or activated by an evenly distributed protease, triggers the protease cascade that produces the Toll ligand. A uniformly distributed inhibitor of protease activity is also involved in determining the final level of the Toll ligand that is produced. The combination of this inhibitor and a protease cascade capable of amplifying a weak ventral cue could therefore transform a moderate asymmetry in the distribution of nudel into a localized source of the Toll ligand. While no such inhibitor has yet been identified, protease inhibitors are important regulators of proteases in other biological processes (Hecht and Anderson, 1992). We cannot rule out the alternative possibility that a ventrally localized cofactor is responsible for local activation of the protease function of nudel. In either case, the local

production of the Toll ligand, which defines embryonic dorsoventral polarity, critically depends on the ability of nudel to organize a zymogen activation complex and to initiate a protease cascade.

Experimental Procedures

Fly Stocks

The wild-type stock was Oregon R. Some of the recessive alleles of *nudel* (*ndl*) and the *pipe* allele (*pip*) used in our studies were isolated in screens for mutations causing female sterility (Anderson and Nüsslein-Volhard, 1984); other *ndl* alleles were isolated in screens for mutations that fail to complement another *ndl* allele (D. Morisato and K. V. Anderson, personal communication). The recessive alleles of *fs(1)K10*, *gurken* (*grk*), *torpedo* (*top*), and *windbeutel* (*wind*) were provided by T. Schüpbach (Schüpbach, 1987; Schüpbach et al., 1991; Neuman-Silberberg and Schüpbach, 1993). The P element lines 371 and *l(3)3844* were provided by W. Gehring (Gehring et al., 1984) and T. Laverty (National Institutes of Health Genome Center and Howard Hughes Medical Institute), respectively.

Determination of the Temperature-Sensitive Period of *nudel*

For temperature downshift experiments, ndl^{9}/ndl^{M6} females were first placed at the nonpermissive temperature of 29°C for 10 days and provided with fresh males every other day. Beginning at 5 hr before shifting the flies to the permissive temperature of 18°C and for 72 hr afterwards, eggs from these females were collected at various times and allowed to develop at 18°C for 5 days. The oogenic stage at time of downshift was deduced by adjusting the duration of oogenic stages at 25°C as described by David and Merle (1968) for slower development at 18°C (Powsner, 1935).

Two distinct temperature upshift experiments were performed. In the first, ndl^{9}/ndl^{M6} females reared at 18°C were shifted to 29°C. Then at various times, eggs were collected from these females over a 1 hr interval and allowed to develop at 29°C for 3 days. The results are shown as the negative time points in Figure 2B. In the second experiment, mutant females remained at 18°C, while only their eggs were shifted to 29°C. Following 1 hr of egg collection at 18°C, eggs were shifted to 29°C at various times to continue development for 3 days. The results are shown as the positive time points in Figure 2B.

For each experiment, we counted the newly hatched larvae and the unhatched eggs (250–1000 per time point) during the 3–5 day period following egg collection to calculate the percentage of eggs that hatched. To confirm that the inability to hatch was due to dorsalization of embryos, cuticles of the unhatched embryos were examined (Wieschaus and Nüsslein-Volhard, 1986).

Generation and Analyses of Chromosomal Deficiencies

Males of the 371 strain, which has a P element with the w^{+} gene in the 65A–B region of the polytene chromosome, were irradiated with 4000 rads of X-rays and then crossed to w^{-}/w^{-} females. From 60,000 F1 progeny, we isolated 41 that lost the w^{+} marker and established 20 stable stocks. Polytene chromosomes from larval salivary glands were examined to determine the cytology of deficiencies. The proximal breakpoints of *Df(3L)CH12* and *Df(3L)CH39* are at 65C1,2 and 65B5, respectively, and the P element line *l(3)3844* has a deletion of the interval 65B1–65C1 adjacent to the P element insertion site at 65C1.

DNA Cloning, Sequencing, and Sequence Analyses

Starting with the cloned *pale* gene at 65B–C provided by W. Neckameyer (Neckameyer and Quinn, 1989), DNA covering the *nudel* locus was cloned from cosmid and phage genomic libraries provided by J. Tamkun (Tamkun et al., 1992). The chromosome walk extended 160 kb from the *pale* locus, which maps 10 kb outside the proximal breakpoint of *Df(3L)CH12*, to the proximal breakpoint of *Df(3L)CH39* (see above).

Segments of genomic DNA from the chromosome walk that hybridized to the 8.4 kb transcript in Northern blots were used as probes to screen ovarian cDNA libraries provided by P. Tolias (Stroumbakis et al., 1994) and G. Waring (Hawley and Waring, 1988). Twenty-four partial cDNAs were isolated; they overlapped to span 8230 nt of the *nudel* transcript length. A cDNA corresponding to the 5′ end of the *nudel* transcript was isolated by PCR from the ovarian cDNA library of G. Waring by using primers based on sequences from the most 5′ cDNA and the library vector.

The *nudel* cDNAs were subcloned into pGEM7(+) (Promega). Internal primers and nested deletions, generated with exonuclease III (Henikoff, 1987), were used for sequencing. Both strands of the composite cDNA representing the entire *nudel* transcript length were sequenced by the dideoxy chain termination method using an automated sequencing system (Applied Biosystems). Sequences were assembled and analyzed with the programs of the Wisconsin Genetics Computer Group. Database searches were performed by using the FASTA program (Lipman and Pearson, 1985).

RNA In Situ Hybridization Analyses

RNA in situ hybridization analyses of whole-mount ovaries were performed according to the protocol of Suter and Steward (1991). Ovaries were dissected into ovarioles before fixation to minimize artifactual asymmetries in hybridization signals, likely to be caused by nonuniform fixation or accessibility of probes to fixed tissue. DNA to detect RNA for the vitelline envelope protein Sv23 was provided by G. Waring (Popodi et al., 1988).

The abnormal morphology of many egg chambers (e.g., bipolar and severely elongated) produced by the strongly and moderately ventralizing alleles grk^{HG21} and grk^{WG} made it difficult to assess the effect of these mutations on *nudel* expression. The analysis of *nudel* expression in *windbeutel* mutants was complicated by the small numbers of homozygous mutant females, due to the reduced viability of these homozygotes, and by the fact that transheterozygous combinations ($wind^{AR51}/wind^{M88}$ and $wind^{AR51}/wind^{M46}$) yielding sufficient numbers of females produced weakly dorsalized embryos.

Nucleic Acid Techniques

Adult genomic DNA and total ovarian RNA were isolated as described by Levis et al. (1982) and by Chasan and Anderson (1989), respectively. Isolation of poly(A)$^{+}$ RNA by oligo(dT) cellulose chromatography, Southern blots, and Northern blots of 7% formaldehyde, 0.8% agarose gels were done according to the protocols of Sambrook et al. (1989). Digoxigenin-labeled DNA probes for Southern blots, Northern blots, RNA in situ hybridizations, and library screenings were prepared by random priming according to instructions in the Boehringer Mannheim Genius kit. Probes were detected with anti-digoxigenin antibody conjugated to peroxidase. Southern and Northern blots were visualized with enhanced chemiluminescence (Amersham).

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GenBank Accession Number

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